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PROJECT TITLE: Tobacco Physiology and Biochemistry
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I. LOW NICOTINE STUDY

A. Objective: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. Status: PM extracts from the ammonium sulfate stage were processed through the two column series of phenyl-Sepharose and DEAE-Sepharose columns. The specific activity of the pooled fractions after the DEAE-Sepharose stage was > 600 units for one preparation. The pooled fractions from the DEAE-Sepharose column following a second series was separated into two parts in which the specific activities were found to be > 300 and > 600 units (1,3,6). A unit is equivalent to a nmole of S-adenosylmethionine converted per mg protein in 30 minutes at 30°C.

A number of affinity support media were further tested for possible use in the purification of PMT; these included ω-aminohexyl Sepharose 4B (AHS), ω-aminopropyl Agarose (APA) and the ω-aminoethyl Agarose (AEA) materials (5,6). Thirty ml of a PMT sample (0.2 mg/ml) was applied to the AHS column in an attempt to concentrate the PMT; however, the elution volume upon removal of PMT with 1 M NaCl was 10-12 ml, indicating that this method is not appropriate to concentrate PMT. Only 15% of the PMT applied to the APA column was eluted upon addition of a 0-30 mM putrescine gradient. More emphasis was therefore concentrated on the AEA column. PMT activity appeared to be separated from the majority of the protein near the beginning of the salt gradient (0-50 mM and 0-100 mM NaCl) with this medium. Moreover, either washing with 10 mM NaCl or 5 mM putrescine was found to be sufficient to elute bound PMT from this support medium. Six mg of protein was determined to be the maximum loading of a 3 ml bed volume column (5,6). Several protein bands, however, were still observed after examination of the PMT sample eluted with putrescine by SDS-PAGE (3,5,6). Work is continuing to find conditions ("affinity") to elute PMT more specifically.

A number of other affinity gels (prepared by Dr. W. P. Hempfling) coupled to N-methylputrescine (NMP) were also examined. PMT was bound to the NMP-aminoethyl-1,6-diaminohexyl Agarose and NMP-CH Sepharose 4B columns. However, no PMT activity was eluted with gradients of the substrates, SAM (0-5 mM) or putrescine (0-30 mM). PMT activity was eluted in an apparent ion exchange mode upon additional application of a 0-500 mM NaCl gradient. The PMT activity eluted with the majority of the protein in the 250-300 mM NaCl concentration range (4). Inconclusive results were obtained from a column in which NMP was coupled to the aminoethyl Agarose support medium (4).

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Further examinations of S-adenosyl-homocysteine (SAH) linked to the 1,6-diaminohexane Sepharose support medium were also conducted (prepared by Dr. W. P. Hempfling) (2). Initial examination showed that some PMT could be eluted upon application of a putrescine gradient (0-50 mM); however, the elution of PMT appeared to be non-specific (possible ion-exchange mode) (2,3). More recently, some PMT was found to be eluted with 1 mM SAM and the remainder upon application of 500 mM NaCl. The SDS-PAGE profile of the PMT sample eluted with 1 mM SAM showed a relatively clean profile with a major band having an apparent molecular weight between 55 and 65 kDa whereas the PMT sample eluted with salt was highly contaminated with a number of protein bands (2,3). These results suggest that sites exist on the affinity material which can bind to PMT in an ion-exchange as well as affinity mode. New gels are being prepared with the use of ethanolamine to reduce the number of ion-exchange sites.

Native gel (ELFE) examination of PMT was also continued. The gel with 10% acrylamide and 10% glycerol appears to give the best results; elution time was reduced considerably with 12% acrylamide (3). Various concentration methods (Amicon, Centriprep and Lyphogel) were also examined. The Amicon and Centriprep methods appear to be the most useful at this time (3).

C. **Plans**: To block ion-exchange sites on affinity matrices for purification of PMT. To focus on the elution of PMT from the affinity matrices, by eluting PMT with substrate conditions. To examine possible increase in the resolution of the native gel (Canalco) system to purify PMT by increasing the length of the gel. Continue ^{3}H-SAM labeling studies. Obtain PMT-active samples for application to affinity and native gel.

D. **References**:

1. Dunn, R. L. Notebook No. 7899.
2. Malik, V. Notebook No. 8542.
3. Davies, S. Notebook No. 8694.
4. Yu, T. Notebook No. 8381.
5. Mooz, E. D. Notebook No. 8599.
6. Crockett, E. Notebook No. 8563.

II. ALTERNATE HUMECTANTS (PG/G-FREE SHEETS/CIGARETTES) (1)

A. **Objective**: To produce an acceptable full-flavored cigarette which is PG/G-free by the end of 1988 and to send it and a suitable control cigarette out as a POL test.

B. **Results**: A request for another PG/G-free cigarette model has been made to the Flavor Development Division personnel (2).

C. **Plans**: The second PG/G-free cigarette model will be evaluated analytically and subjectively. Control feedstocks and PG/G-free

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RL, RCB, ESB, and DIET will be re-evaluated analytically, microbiologically, and subjectively prior to making PG/G-free POL cigarettes.

D. **References:**

1. Mooz, E. D. Notebook No. 8599.

2. Ruziak, S. Personal Communication to E. Mooz. 1988 October 19.

III. ALTERNATE HUMECTANTS (GLYCERINE-FREE SHEET/CIGARETTES) (1)

A. **Objective**: To produce an acceptable glycerine-free (G-free) cigarette (domestic Marlboro-type) for a POL test by the end of the first quarter of 1989.

B. **Results**: The G-free program has been put on hold pending the outcome of the PG/G-free effort.

C. **Plans**: There are no further plans at this time.

D. **References:**

1. Mooz, E. D. Notebook No. 8599.

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